

The Role of the Biofunctionalized Silica Nanospring Surface for Selective Biosensing

Yukta P. Timalisina^{*1}, Josh R. Branen², Jeremy Eilers³, Blaise Alexis Fouetio Kengne⁴, D. Eric Aston⁵, Giancarlo Corti⁶ and David N. McIlroy^{*7}

^{1,2,3,4,7}Department of Physics, University of Idaho, 875 Perimeter Drive MS 83844-0903, Moscow ID 83844-0903, USA

⁵Department of Chemical and Materials Engineering, University of Idaho, Moscow, ID 83844-1021, USA

⁶Department of Mechanical and Materials Engineering, Washington State University, Pullman, WA 99164, USA

^{*1}timaly@rpi.edu; ²jbranen@biotracking.com; ³eile9457@vandals.uidaho.edu; ⁴foue3398@vandals.uidaho.edu;

⁵aston@uidaho.edu; ⁶luigi.corti@wsu.edu; ^{*7}dmcilroy@uidaho.edu

Abstract

We have addressed the challenges by integrating an immunoassay technique, which is one of the most powerful techniques because of the specificity and selectivity of antigen-antibody interaction, into a biosensor constructed with vertically-aligned (silica) nanosprings (VANS). The unique characteristics of VANS, such as biocompatibility, large surface-to-volume ratio, etc., facilitate selective sensing and miniaturization. Silane chemistry is used to functionalize VANS surfaces to covalently link a probe layer onto the VANS, providing better control over the concentration and orientation of the attached biomolecule. A mouse immunoglobulin G (IgG), which is sandwiched in between two layers of a goat antimouse IgG, binds the goat antimouse IgG, and is detected by the changes in the impedance of the biosensor. The selectivity of the sensor has been verified using rabbit IgG as a control. The experiments reveal that the response of the VANS sensor is solely due to the interaction of the target molecule, i.e. mouse IgG, with the probe layer, i.e. goat antimouse IgG (GaM IgG). The change in impedance indicates that the addition of antibodies impedes ionic motion, thereby changing the effective dielectric response of the VANS-biomolecular medium.

Keywords

Immunosensor; Antibody; Impedance; VANS; Electric Double Layer

Introduction

The demand for selective and stable biosensors in areas such as medical diagnostics, bio-defense, and food safety, is greater than ever before. Despite extensive exploration of many types of nanomaterials for use in biosensors, the construction of nanomaterial-based biosensors still remains a challenge. To this end, immunoassays may be the key to realizing nanomaterials-based biosensors. The immunoassay is

one of the most powerful analytical tools due to the specificity and selectivity of antigen-antibody interaction (Fu et al.). The basic principle of the immunoassay technique involves the molecular recognition between an antibody and a specific antigen, and this makes it naturally very useful for clinical analysis (Hartwell & Grudpan). However a simple, cheap and robust immunoassay is needed because conventional immunoassays are labor intensive, expensive and time consuming and require large pieces of equipment for detection (Maeng et al.). The development of biosensors (e.g. immunosensors) through integration of biological molecules with specifically designed physical transducer surfaces is one way to overcome this limitation. In addition, the use of nanomaterials within these designs holds promise that the unique characteristics of these materials may allow novel sensitivity and miniaturization possibilities. However, all nanomaterials do not fit the bill for use in biosensors. Vertically-aligned (silica) nanosprings (VANS) have a number of desirable properties that are well suited as a platform upon which to build a biosensor. Because they are silica (glass) they are biocompatible, which is an advantage were they to be used in an in vivo sensor (Slowing et al.). Their insulating glass composition makes them ideal for impedance based biodetection. VANS have a large surface area of the order of 400 m²/g or greater, which facilitates miniaturization without sacrificing the number of receptors.

The immobilization of biomolecules onto transducer surfaces is the first and foremost step for the successful development of fully functional electrical-based biosensors. Several immobilization techniques on solid

support have been reported (Nisnevitch; Kusnezow & Hoheisel). Some examples are physical entrapment of biomolecules (Cosnier; Ruan et al.), the formation of surface functional groups (carboxyl, amine, etc.) through various chemistries such as silane to covalently link biomolecules to the surfaces (Liu; Popovich, Yen, & Wong; Moon et al.), or gel coatings (Ray, Feng, & Tachikawa; Lee et al.). Biosensors based on physical adsorption avoid the additional complexities of functionalization and binding chemistry. Therefore, these biosensors are simple, cheap and easy to handle. However, the drawback to physical adsorption is that the distribution of enzyme molecules is neither uniform nor orientation specific, can be unstable, and tends to leach with time. Furthermore, the chemical interaction tends to partially denature the activity of the proteins. In contrast, the covalent attachment of functional proteins to a solid support is site specific and stable (Shriver-Lake et al.). Therefore, covalent attachment of probe/target onto the VANS functionalized surface is preferred, not only to avoid desorption of the probe layer but also to facilitate better control the number and orientation of the attached probe biomolecule.

Despite many detection methods, capacitive-based (Berggren & Johansson; Labib et al.; Berggren, Bjarnason, & Johansson) and impedance based detections (Ionescu et al.), for example, the construction of stable and selective biosensors still remains a challenge (Feng et al.). Some major challenges can be overcome through the construction of highly selective and stable biorecognition layer where mouse IgG is sandwiched in between two layers of goat anti-mouse IgG on the functionalized-VANS surface. The specific and selective binding of the target (mouse IgG) to the immobilized probe layer (GaM IgG) inhibits the diffusion of ions to and from the mat thereby changing the electrical properties, such as capacitance, resistance, etc., of the sensor. The advantage of the proposed technique, which aims at a reliable and easy procedure, enables detection of biomolecules desired stability, selectivity and sensitivity, all without the use of labelling.

Materials and Methods

Device Fabrication

The sensor is a parallel plate capacitor with upper and lower electrodes consisting of indium tin oxide (ITO) coated glass slides, where VANS has been grown on

the bottom electrode surrounded by the test fluid. The details of the device fabrication process have already been described (Timalsina, Oriero, et al.; Senturia). The technology used to grow the nanosprings has been previously reported (Wang et al.; Norton & McIlroy; McIlroy et al.). Figures 1 (a) and (b) show scanning electron microscopy (SEM) images of VANS. The thickness of the VANS is $30 \pm 2 \mu\text{m}$.

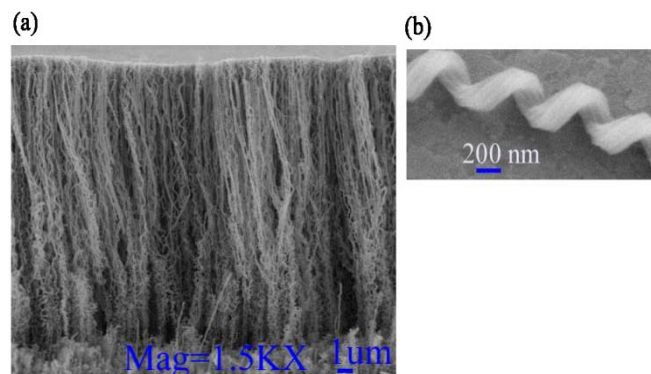


FIG. 1 SCANNING ELECTRON MICROSCOPY (SEM) IMAGES OF (A) VANS MAT AND (B) A SINGLE NANOSPONG

VANS Surface Functionalization

Bottom electrodes with VANS grown on them were dipped in 10% aminopropyltriethoxysilane (APTES) prepared in absolute ethanol for 10 minutes under dry nitrogen. The electrodes were washed three times in absolute ethanol and were heated to 110°C to allow condensation of the silanols from the aminosilane. The aminosilanized VANS surface was treated in 5% glutaraldehyde (GA), a homobifunctional crosslinker, prepared in de-ionized (DI) water for 30 minutes and then washed in DI water. An amine functional group reacts with the aldehyde group at one end of GA forming imine bond, leaving an aldehyde group at the other end to react with the primary amine groups of the antibody.

Surface Characterization Technique

X-ray photoelectron spectroscopy (XPS) was used to confirm the presence of chemical functional groups and the chemical bonding on the functionalized VANS surface. The measurements were performed in an ultrahigh vacuum chamber equipped with an Omicron model EA 125 hemispherical electron energy analyzer with a resolution of 0.025 eV . The base pressure prior to spectra acquisition was 1.5×10^{-10} Torr. The $\text{MgK}\alpha$ emission line (1253.6 eV) was the X-ray energy. The sample was grounded in conjunction with a 550 eV electron beam from an electron flood

gun to avoid charging of the VANS. All the spectra were collected at room temperature and normal emission. The binding energy scale was calibrated using the peak energy of C 1s (284.70 eV).

Immobilization Protocols

A 1X phosphate buffer (PBS) (cat# 20012043) was purchased from Invitrogen (Carlsbad, California) and was used without purification. A 1 mg/ml fluorescein iso-thiocyanate (FITC)-labeled goat anti-mouse IgG (cat# 610-102-121), a 1 mg/ml unlabeled goat anti-mouse IgG (cat# 610-101-121), a 10 mg/ml mouse IgG (cat# 010-0102) and a 10 mg/ml rabbit IgG (cat# 011-0102) were purchased from Rockland Immunochemicals (Gilbertville, Pennsylvania) and bovine serum albumin (BSA) was purchased from Amresco (Solon, Ohio). Antibody solutions were used without purification and each of these solutions was diluted in 1X PBS to make concentrations of each of 1 $\mu\text{g/ml}$.

The Immobilization protocol for each step of immobilization is shown in Figure 2 (Yuan, Mullett, & Pawliszyn; Arnoys; Clark). A goat anti mouse (GaM IgG I) antibody was immobilized to APTES-GA modified surface through the reaction between the amine group on the protein and the aldehyde group on the surface (i.e. via imine bond). BSA, which reacts with any remaining free aldehydes of the surface, reduces additional surface adsorption. A 1 $\mu\text{g/ml}$ solution of BSA was added to the APTES-GA modified surface after washing the sensor several times with PBS. A mouse IgG or rabbit IgG (in control) was

captured from the solution phase through a reaction with the APTES-GA-GaM IgG I surface. The mouse IgG binds to GaM IgG on APTES-GA-GaM IgG I surface. Following a thorough washing, another layer of GaM IgG II was introduced to the VANS surface in solution phase. This molecule reacts with the surface only if there is mouse IgG captured in the previous step of immobilization. Each step of immobilization took two hours followed by washing the sensor surface thoroughly with PBS.

Image Acquisition and Analysis

An Olympus BX-51 fluorescence microscope fitted with a FITC filter and an Optronics MagnaFIRE SP CCD camera was used for image acquisition. The captured images were analyzed using the image processing toolbox available in MATLAB. Image histograms represent the number of pixels for each level of brightness by analyzing a pixel section of 1280×1024 . Since the FITC has an emission peak in a frequency corresponding to a green color, only the green component of the image was analyzed. These histograms were used to confirm the presence of GaM IgG and mouse IgG (or rabbit IgG in control) in VANS sensor.

Measurement Method

The test solutions consisting of FITC labeled GaM IgG or unlabeled GaM IgG, mouse IgG (or rabbit IgG) was introduced into the VANS sensor. Each of the

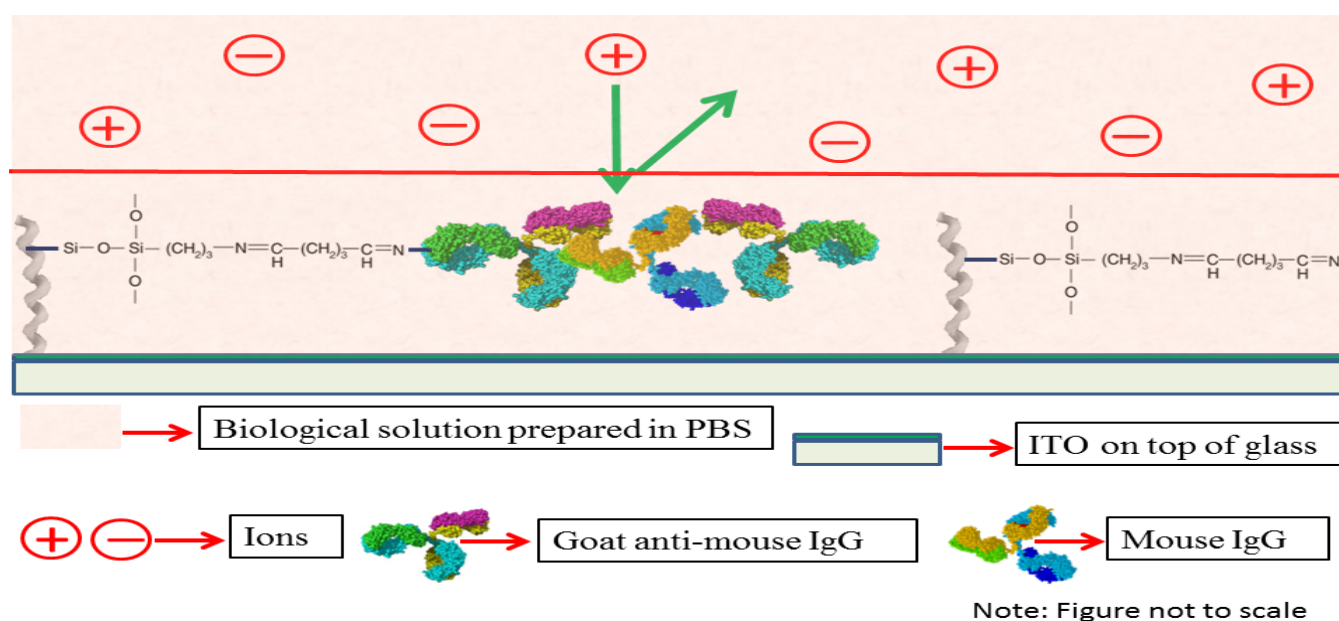


FIGURE 2 SCHEMATIC OF IMMOBILIZATION OF ANTIBODIES TO FUNCTIONALIZED VANS SURFACE

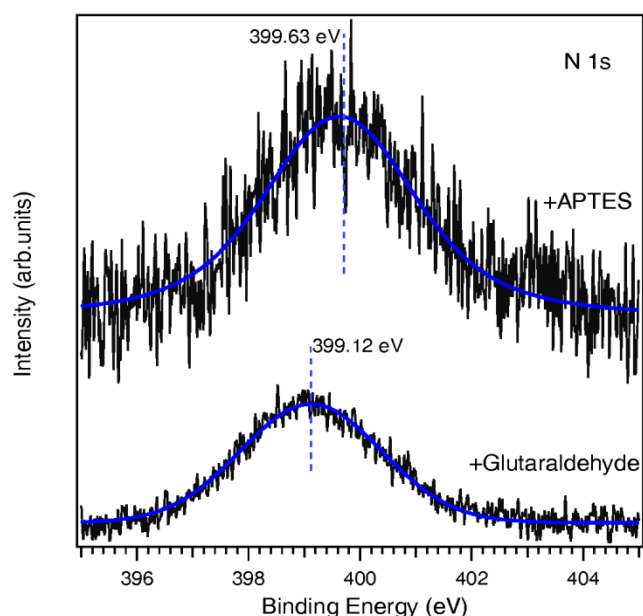


FIGURE 3 N 1S CORE LEVEL STATE OF THE FUNCTIONALIZED VANS SURFACE AFTER IMMOBILIZATION OF APTES (UPPER CURVE) AND APTES-GA (LOWER CURVE)

VANS sensors were tested with PBS before immobilization of GaM IgG I. Following the immobilization of FITC labeled GaM IgG on VANS

functionalized surface for 2 h, impedance and fluorescent measurements were carried out to confirm the presence of FITC labeled GaM IgG on the sensor surface. Then unlabeled GaM IgG was immobilized for 2 h and washed through with PBS. Mouse IgG was attached to GaM IgG modified surface. FITC labeled GaM IgG was added to unlabeled GaM IgG -mouse IgG modified surface. Impedance measurements were carried out in each step of immobilization. Finally, fluorescent measurements were performed to confirm the presence of GaM IgG. Test solutions for control experiments consisted of the same solutions except that mouse IgG was replaced by rabbit IgG. Spectra were measured using a VersaSTAT3 with frequency response analyzer and software to measure the impedance from 1 Hz to 1 MHz with sine wave amplitude of 10 mV applied. We modeled the measured impedance response vs. frequency of the VANS sensor with a resistor-inductor-capacitor (RLC) circuit methodology. The values of the fitted elements of the equivalent circuits were averaged and their standard deviations were calculated.

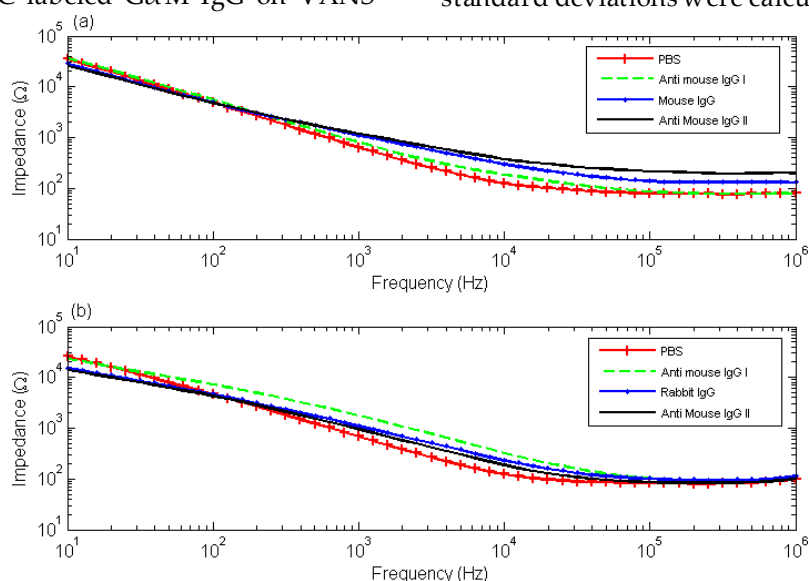


FIGURE 4 IMPEDANCE SPECTRA MEASURED AT 10 MV APPLIED VRMS AFTER THE SUBSEQUENT ADDITION OF PBS, GAM IGG I, MOUSE IGG (OR RABBIT IGG) AND GAM IGG II: IMPEDANCE MAGNITUDE PLOT AS A FUNCTION OF FREQUENCY FOR (A) THE VANS SENSOR AND (B) THE CONTROL

Results and Discussion

Displayed in Figure 3 are the XPS spectrum of the N 1s core level states of APTES and GA functionalized VANS. A complete characterization of silanized silica surface using XPS has been reported elsewhere (Libertino et al.). The N 1s peaks for APTES-VANS and GA-APTES-VANS surfaces were centered at 399.63 eV and 399.12 eV, respectively. This is consistent with the previously reported values for the

silanized silica surface (Libertino et al.). The N 1s signal from the APTES is suppressed in the GA-APTES-VANS surface, as compared to APTES-VANS surface. GA, which is devoid of nitrogen, forms a uniform layer on the APTES subsequently attenuating the N 1s signal of the APTES.

The frequency dependent impedance spectra with the subsequent addition of the different biological solutions (GaM IgG I, mIgG and GaM IgG II) are

shown in Figure 4 (a). Above 100 Hz the average impedance increases by 66% with the addition of GaM IgG II relative to PBS. However, below 100 Hz the impedance decreases. The impedance continues to decrease with the subsequent addition of additional biological solutions (GaM IgG I, rabbit IgG and GaM IgG II) in its control, as shown in Figure 4 (b). This indicates that neither GaM IgG I nor the second probe layer GaM IgG II binds to rabbit IgG and the impedance change is solely due to the increasing concentration of the solution. Image histogram plots obtained from fluorescent measurements of the VANS sensor and its control are shown in Figures 5 (a), (b) and (c). Detailed examination of the intensity histogram of the GaM IgG I -VANS sensor [Figure 5 (a)] reveals peak intensity at 0.2, confirming that GaM IgG I is immobilized on the VANS surface which is consistent with the impedance measurements [Figure 4 (a)]. Figures 5 (b) and (c) are the intensity histogram for unlabeled GaM IgG I-mouse IgG- FITC labeled GaM IgG II and GaM IgG I-rabbit IgG- FITC labeled

GaM IgG II, which reveal peaks at 0.3 and 0 respectively, confirming that the observed impedance changes arise from the specific interaction between GaM IgG and mouse IgG, thereby demonstrating the selective binding.

The impedance magnitude spectra for the VANS sensors and controls are interpreted with a previously reported model equivalent circuit [(Timalsina, Branen, et al.)], which is shown in Figure 6. Three individual VANS sensors and controls were analyzed with this circuit in order to arrive at average values of the elements as a function of the specifics of the biological solutions. The results are summarized in Tables 1 and 2.

VANS surfaces have both silanol (Si-OH) and siloxane (Si-O-Si) groups and are electrically charged by releasing protons according to the following equation (Lorenz et al.):

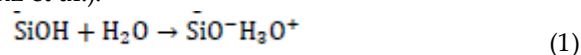


Table 1 AVERAGE VALUE OF THE CIRCUIT ELEMENTS IN THE EQUIVALENT CIRCUIT SHOWN IN FIGURE 6 OBTAINED FROM FITTING THE EXPERIMENTAL IMPEDANCE SPECTRA AS A FUNCTION OF PBS, GAM IGG I, MIGA AND GAM IGG II FOR THE VANS SENSOR

Solution	C ₁ (μF)	C ₂ (nF)	R _s (Ω)	R ₁ (kΩ)
PBS	2.08±1.71	433±67	87±8.0	3.67±0.44
GaM IgG I	0.38±0.04	270±70	99.7±8.5	5.60±0.78
mIgG	0.48±0.12	177±14	131.0±5.3	6.63±0.81
GaM IgG II	0.55±0.08	117±20	171.3±20.7	8.00±0.36

Table 2 AVERAGE VALUES OF THE CIRCUIT ELEMENTS IN THE EQUIVALENT CIRCUIT SHOWN IN FIGURE 6 OBTAINED FROM FITTING THE EXPERIMENTAL IMPEDANCE SPECTRA AS A FUNCTION OF PBS, GAM IGG I, RABBIT IGG AND GAM IGG II FOR THE CONTROL OF VANS Sensor

Solution	C ₁ (μF)	C ₂ (nF)	R _s (Ω)	R ₁ (kΩ)
PBS	2.13±0.92	625±143	75±6	5.00±0.82
GaM IgG I	1.18±0.27	250±122	85±7	6.25±0.53
Rabbit IgG	4.50±2.45	340±131	87.5±2.0	3.50±1.20
GaM IgG II	4.88±2.14	350±122	82.5±6.1	3.00±1.20

The charged VANS surface attracts opposite ions in the solution. Consequently, two equal and oppositely-charged layers result in an additional electric double layer at the solution/VANS interfaces. This electric double-layer capacitance (C₂) dominates the circuit response in the frequency range of 100 Hz to 10 kHz and increases with the addition of subsequent biological solutions. However, C₂ decreases from 625

nF to 250 nF in the control [Table 2] due to the binding of GaM IgG I. Then C₂ increases to 350 nF with the immobilization of GaM IgG II in control. This shows the selectivity of the sensor (i.e., GaM IgG only binds to mouse IgG and not to rabbit IgG). Therefore, the increase in capacitance C₂ with the addition of rabbit IgG and GaM IgG II is due to the increase in buffer concentration only.

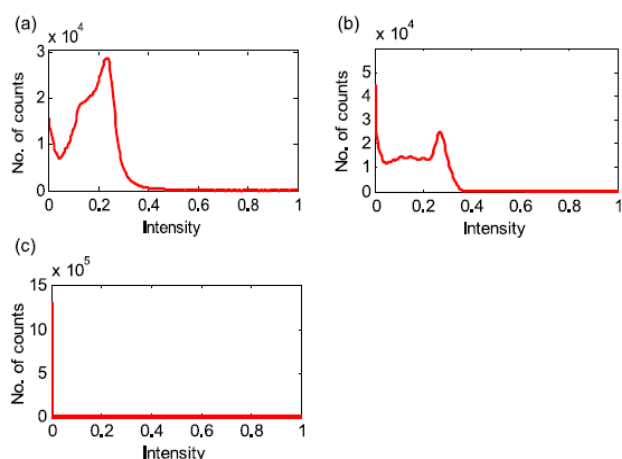


FIGURE 5 INTENSITY HISTOGRAMS OF FLUORESCENCE EMISSION OF (A) GAM IGG I, (B) MOUSE IGG AND (C) RABBIT IGG

This capacitance can be described as built from several capacitances in series. The first capacitance constitutes the functionalized-VANS insulating layer, C_{ins} . The second capacitor, C_{p1} , includes the first probe layer and any contribution from the Stern layer. The third capacitor, C_t , includes the target layer and part of the contribution from the diffuse layer. The fourth capacitor, C_{p2} , is described by the second probe layer and the concentration dependent diffuse layers, extending out into the bulk solution. Therefore, the total capacitance C_2 can be written as

$$\frac{1}{C_2} = \frac{1}{C_{ins}} + \frac{1}{C_{p1}} + \frac{1}{C_t} + \frac{1}{C_{p2}} \quad (2)$$

From equation (2), one can tell that total capacitance with the addition of biological solutions. In addition, probe (GaM IgG) and target (mouse IgG) binding displaces water and ions from the surface upon binding. The permittivity ϵ_r of biomolecule is in the range of 2–5 versus 80 for water (Daniels; Pourmand). As a result, permittivity of the system decreases which results in the decrease in capacitance.

Solution resistance within VANS (R_1) dominates in the frequency range between 100 Hz–10 kHz. R_1 arises from the effective electrostatic size of the channels between neighboring VANS. The probe/target binding decreases the effective channel size, from an electrodynamics standpoint, thereby hindering the diffusion of ions to and from the ITO electrode plane. Therefore, R_1 increases with the addition of biological molecules onto the VANS sensor surface.

The solution resistance (R_s) predominates in the frequency range of 10 kHz–10 MHz and is independent of the driving frequency. R_s depends on bulk solution concentration. The value of R_s increases with

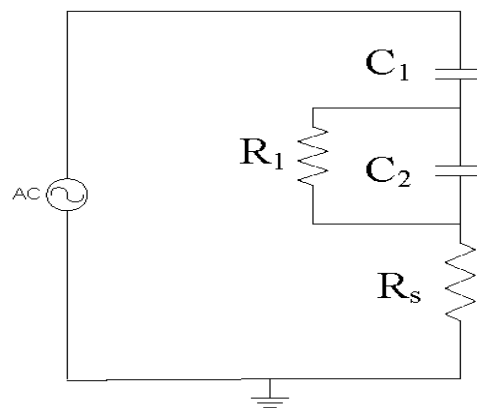


FIGURE 6 A MODEL EQUIVALENT CIRCUIT

probe/target binding due to the increase in the thickness of the functional layer on the electrodes. However, there is not much change in solution resistance with addition of rabbit IgG and GaM IgG II in control.

Electric double-layer capacitance (C_1) corresponds to the double layer formed at the solution-electrode interface and dominates in the frequency range below 100 Hz. C_1 depends on thickness of double layer and dielectric constant of materials in between two layers provided area of the layer constant. Closer examination of Table 1 reveals that C_1 decrease with the addition of biological solutions. The increase in C_1 values in control is greater by an order of magnitude in control compared to the VANS sensor (see Table 2). Isoelectric point (PI) of GaM IgG, mIgG and rabbit IgG are 6.8 (Mak et al.), 9.5 (Lee, Huang, & Ayres) and 8.6 (Bollen & Hau) respectively. Therefore, GaM IgG, mouse IgG and rabbit IgG are expected to acquire negative, positive and positive charges, respectively, in PBS of pH 7.2. The total negative charge on the VANS surface increases upon probe (i.e., GaM IgG) binding. The total charges on the VANS- GaM IgG I surface are neutralized by opposite charges of target (i.e., mouse IgG) upon mouse IgG immobilization. The ions experience almost no net electrostatic forces on them from the neutralized surface. Therefore, they move almost freely towards the electrode, which increases charge density in the EDL. This results in a decrease of EDL thickness thereby increasing the capacitance upon probe/target binding.

The variations of the fitted values for PBS and GaM IgG in VANS sensors and controls are due to slight variations among sensors (i.e., variations in channel size, mat thickness, pore size, etc.) that arise from variability of the VANS. In turn, this can produce a change in the surface distribution of the functional groups attached to the aminosilanized surface. Even

though a very careful and systematic procedure for silanizing the surface of the VANS was followed, when working with nanomaterials there will always be some variability in the surface distribution of the functional groups. Therefore, it is the trends and not the absolute values that define the sensor response. However, this variability at low concentrations will need to be studied in order to determine the absolute sensitivity of the VANS biosensor. As discussed above, our biosensor design exhibits excellent selectivity, stability and sensitivity compared to many biosensors reported in the literature that have shown poor, or no, selectivity/specificity. This work lays down a foundation upon which further development can be built. The incorporation of microchannels of VANS into a microfluidic sensor design takes advantage of several intrinsic characteristics of microfluidics, such as laminar flow for improved delivery, low consumption of costly reagents, short reaction times for analysis, portability, and versatility in design. The results of this study show that VANS biological sensing is the most sensitive in the frequency range from 100 Hz-1 MHz. Consequently, one can extend the sensitivity of the electrical measurements using lock-in detection techniques, which is extremely sensitive to AC signals in the bandwidth frequency of 100 Hz-1 MHz. With the aforementioned improvements in VANS sensor design and measurement techniques, the next step will be to explore their limits of sensitivity and selectivity, as well as their dynamic range, thereby launching them into real-world commercial applications.

Conclusions

Silane chemistry was successfully employed to covalently functionalize VANS surfaces with antibodies. Impedance spectroscopy was used to monitor layer-by-layer deposition of antibodies onto the functionalized VANS surface. This study demonstrated the feasibility of covalently linking GaM IgG for sandwich immunosensor applications. In addition, the interaction between GaM IgG and mouse IgG demonstrated the stability, selectivity, sensitivity and specificity of binding. Based on extracted numerical values of the equivalent circuit elements of the VANS sensor in Table 1, it was shown that C_2 , R_1 and R_s are the most sensitive elements of the equivalent circuit, which can be used as a secondary form of detection. It was determined that above 100 Hz the VANS-based sensors exhibit a greater magnitude of change between successive bio-layers relative to the controls, which indicates that the addition of

biomolecules inhibits the diffusion of ions and changes the effective dielectric response of the VANS-biomolecular medium. In addition, VANS show promise for the development of highly sensitive, selective, low cost, and stable label-free biosensors.

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Yukta P. Timalsina earned his B.S. in Physics from Tribhuvan University, Kathmandu, Nepal in 2001 and his M.S. in physics from the University of Idaho, Moscow, Idaho in 2009 while concurrently earning his PhD in Physics also at the University of Idaho completed a few years

later in 2012.

He began a career in higher education as Post-Doctoral Research Associate at Rensselaer Polytechnic Institute in 2012, where he is currently working in the same position in the center of Integrated Electronics. His research interests include advanced thin films and metal interconnect technology, x-ray crystallography, nanoscience and

nanotechnology, and chemical and biological sensing.

Dr. Timalsina is a member of American Physical Society.

Josh R. Branen earned his B.A. in Psychology from the University of Montana, Missoula, MT in 1998 and his M.Ed. in counseling from the Northern Arizona University, Flagstaff, AZ in 2000 and a Ph.D. in microbiology, molecular biology and biochemistry from the University of Idaho, Moscow, ID in 2006.

He began his research career at the University of Idaho as a Research Scientist in the Biosensor and Nanotechnology Applications Laboratory. He is currently the Chief Executive Officer and Director of Product Development at BioTracking LLC in Moscow, ID. His research interests include molecular and immunodiagnostics, biosensor applications, nanoscience and nanotechnology, and diagnostic platform development.



Jeremy K. Eilers earned B.A. in Physics from Walla Walla University (College Place, Wa) in 2010.

He is currently a graduate student at the Department of Physics, University of Idaho, Moscow, Idaho.



Blaise Alexis Fouetio Kengne received Physics teaching degrees from Ecole Normale Supérieure of Yaounde, Cameroon in 1993 and 2002, B.Sc. and M.S. in Physics from the University of Yaounde I, Cameroon in 1993 and 2004 respectively.

He is currently a Physics PhD candidate at the University of Idaho, Moscow, Idaho. His research focuses on surface analysis of nanospring-based catalysts for biofuels production.



D. Eric Aston earned his B.S. in chemical engineering from the University of Idaho, Moscow, ID in 1995 and his M.S. in Physics from the University of Washington, Seattle, WA in 2000 while concurrently earning his PhD in chemical engineering also at the University of Washington completed a few

months later in 2001.

He began a career in higher education as Assistant Professor of Chemical Engineering at the University of Idaho-Moscow in the summer of 2001, where he is currently Associate Professor of Chemical Engineering and Adjunct Associate Professor of Materials Science and Engineering in the Department of Chemical and Materials Engineering. His research interests include colloids and interfacial phenomena, quantitative atomic force microscopy, nanoscience and nanotechnology, and Raman spectroscopy.

Dr. Aston is a member of the Society for Applied Spectroscopy.



Giancarlo Corti earned his B.S. in Mechanical Engineering from The Escuela Politécnica del Ejército, Sangolquí Ecuador, in 1999; he later obtained his M.S. and his Ph.D. in Mechanical

Engineering from the University of Idaho in 2001 and 2006 respectively.

He joined as a postdoctoral fellow in Dr. McIlroy's research group at the Department of Physics, University of Idaho in 2006 until he joined GoNano Technologies as Manager of Research and Development, in 2008. Currently, He is a Clinical Associate Faculty at Washington State University. He has made several contributions in the areas of acoustics in fluids, MEMS, NEMS, hierarchical nanostructures, biosensors and design and development of equipment and procedures for mass production of nanomaterials.



David N. McIlroy earned his B.A. in Physics from the University of California, Santa Cruz, CA in 1984 and his PhD in Physics from the University of Rhode Island in 1993.

Upon completion of his Bachelor's degree he worked as a quality engineer in the semiconductor industry. He also worked as a project engineer overseeing the building of physical vapor deposition equipment used in semiconductor manufacturing. From 1987-1993 he pursued his advanced degree in Physics. After a postdoc in the Department of Physics at the University of Nebraska-Lincoln, he joined the Department of Physics at the University of Idaho, USA, in 1996, where he is currently Professor and Chair of Physics. His research interests include the synthesis, characterization and application of one-dimension nanostructures.

Dr. McIlroy is a member of the American Physical Society